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Chemosensitization of gemcitabine-resistant human bladder cancer cell line both *in vitro* and *in vivo* using antisense oligonucleotide targeting the anti-apoptotic gene, clusterin

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OBJECTIVE

To characterize changes in clusterin (sCLU-2) expression in bladder cancer cells after continuous treatment with gemcitabine and to determine whether knockdown of sCLU-2 can re-introduce sensitivity of gemcitabine-resistant cells to treatment with gemcitabine.

MATERIALS AND METHODS

A human bladder cancer cell line, UM-UC-3, was continuously exposed to increasing doses of gemcitabine *in vitro*, and a gemcitabine-resistant cell line UM-UC-3R was developed. The role of sCLU-2 in chemoresistant phenotype acquired in both *in vitro* and *in vivo* was then analysed using antisense oligonucleotide targeting the *sCLU-2* gene (OGX-011).

RESULTS

Treatment of parental UM-UC-3 cells (UM-UC-3P) with gemcitabine induced transient up-regulation of sCLU-2 protein. There was a sustained increase in sCLU-2 expression levels in UM-UC-3R compared with UM-UC-3P cells (6.4-fold). Treatment of UM-UC-3R cells with OGX-011 resulted in a dose-dependent and sequence-specific inhibition in sCLU-2 expression. Furthermore, OGX-011 chemo-sensitized UM-UC-3R cells to gemcitabine *in vitro* with a reduction in the concentration that reduces the effect by 50% (IC_{50}) from 100 nM to 10 nM. Tumour volume and the incidence of metastasis in nude mice injected with UM-UC-3R cells was significantly greater than those of nude mice injected with UM-UC-3P cells; however, systemic administration of OGX-011 plus a low dose of gemcitabine significantly suppressed tumour volume and the incidence of metastasis in both groups.

CONCLUSION

These findings suggest that sCLU-2 plays a significant role in the acquisition of chemoresistant phenotype in bladder cancer cells and the knockdown of sCLU-2 using OGX-011 combined with a chemotherapeutic agent could be an attractive approach for advanced bladder cancer through the enhancement of chemosensitivity.

KEYWORDS

clusterin, antisense oligonucleotide, bladder cancer, gemcitabine, drug resistance

INTRODUCTION

Bladder cancer is the second most common malignancy of the genitourinary tract and the fourth or fifth leading cause of cancer-related death of men in Western industrialized countries [1]. Invasive bladder cancer accounts for 20–30% of all newly diagnosed bladder cancers. The standard treatment for localized muscle-invasive bladder cancer is radical cystectomy and pelvic lymphadenectomy, whereas patients with unresectable extravesical involvement or distant metastases are usually treated with cisplatin-based combined chemotherapy [2–6]. Despite the combined use of aggressive therapeutic methods consisting of surgical resection, chemotherapy and/or radiotherapy, invasive bladder cancer has a 5-year survival rate of ≤50% [7]. Thus, there is a pressing need for developing novel therapeutic strategies that improve survival outcomes with reduced toxicity compared with the conventional approach.

Recently, gemcitabine (2′, 2′-difluorodeoxycytidine), a deoxycytidine analogue, has proven activity in several solid tumours [8], including advanced bladder cancer [9,10]. Furthermore, several large randomized phase III clinical studies showed that there were no significant differences in long-term overall and progression-free survivals between patients receiving methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) and those receiving combined chemotherapy using the combined chemotherapy with gemcitabine plus cisplatin (GC) and that GC showed better tolerability and safety profile than MVAC [11,12]. Thus, GC is considered a standard regimen for patients with locally advanced and metastatic bladder cancer.

Despite a reasonable response rate after initial chemotherapy in patients with metastatic bladder cancer, 60–70% of responding patients relapse within the first year, with a median survival of 12–14 months [13]. This limited efficacy may be due to *de novo* drug resistance and/or the development of cellular

drug-resistant phenotype during treatment. Experimental models have helped clarify mechanisms associated with acquisition of chemotherapeutic agents in cancer cells. However, no study has focused on the resistant phenotype of bladder cancer to gemcitabine; therefore, the application of gemcitabine-resistant bladder cancer cells to preclinical experimental model may uncover novel findings for elucidating molecular mechanism of drug-resistance resulting in the development of novel strategies for advanced bladder cancer.

In bladder cancer, previous studies reported that clusterin (sCLU-2) over-expression is closely associated with disease recurrence and progression [14], and that antisense (AS) oligodeoxynucleotide (ODN) targeting the *sCLU-2* gene synergistically enhances the cytotoxic effects of cisplatin, and this combined treatment inhibited tumour growth and metastasis in human bladder cancer models [15]. Many studies have highlighted the important role of sCLU-2 in the development of drug-resistant phenotype in several kinds of cancers [16–19]. However, it has not been characterized whether the development of gemcitabine-resistant phenotype is associated with increased sCLU-2 expression. Therefore, in the present study we initially developed a drug-resistant human bladder cancer cell line by continuous exposure to gradually increasing, clinically relevant doses of gemcitabine. We then addressed the functional role of sCLU-2 during the development of an acquired resistance to gemcitabine and further investigated the efficacy of a combined treatment with gemcitabine and second-generation AS ODN targeting *sCLU-2* gene in an *in vivo* human bladder cancer model.

MATERIALS AND METHODS

For the tumour cell line UM-UC-3 cells, derived from human bladder cancer, were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin.

Gemcitabine was provided by Lilly Research Center Ltd (Indianapolis, IN, USA). Methotrexate, paclitaxel, doxorubicin and cisplatin were purchased from Biolyse Pharma (St Catherines, Ontario, Canada). Stock solutions of chemotherapeutic agents were prepared with PBS to the required concentrations before each *in vitro* and *in vivo* experiment.

To develop the gemcitabine-resistant bladder cancer cell line, parental UM-UC-3 cells (UM-UC-3P) were serially treated in a 10-cm dish with 1 nM of gemcitabine for 1 week ('on' cycle) followed by being without treatment for 1 week ('off' cycle). The concentrations of gemcitabine were gradually increased up to 50 nM during the subsequent treatment of eight 'on' and eight 'off' cycles. Thereafter, these cells were continuously cultured in complete medium supplemented with 50 nM gemcitabine for >20 passages. This cell line, UM-UC-3R, was considered as stably established cell line because the growth rate of this cell line with 50 nM gemcitabine was constant for at least 30 days. UM-UC-3R was used as a gemcitabine-resistant bladder cancer cell line for all subsequent experiments.

For Western blot analysis, samples containing equal amounts of protein (20 μg) from lysates of the cultured cells were subjected to SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked in PBS containing 5% nonfat milk powder at 4 °C overnight and then incubated for 2 h with 1:1000-diluted anti-clusterin goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 1:2000-diluted vinculin mouse monoclonal antibody (Sigma Chemical Co., St. Lous, MO, USA). The filters were then incubated for 1 h with 1:5000 diluted horseradish peroxide-conjugated anti-goat or anti-mouse IgG antibody (Santa Cruz), and specific proteins were detected using an enhanced chemiluminescence Western Blot analysis system (Amersham, Life Science, Arlington Heights, IL, USA). The strength of each signal density was determined using a densitometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

The 2′-*O*-methoxy ethyl-modified AS ODNs used in this study were synthesized as described previously [20]. The sequence of the OGX-011, targeting *sCLU-2* gene corresponding to the human sCLU-2 translation initiation site was 5′-CAGCAGCAGAGTCTTCATCAT-3′ (OncoGenex Technologies Inc., Vancouver, Canada). The sequence of ODN used as a control in this study was 5′-CAGCAGCAGAGTATTTATCAT-3′. Both ODNs displayed a length purity of >95% with a phoshphodiester content of <0.3%. A Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database showed no homology of OGX-011 and control ODN to any other known human genes.

Oligofectamine, a cationic lipid (Invitrogen Corporation, Carlsbad, CA, USA), was used to increase the ODN uptake into the cells. Cells were treated with various concentrations of ODN after preincubation for 20 min with 3 mg/mL oligofectamine in serum free OPIT-MEM (Invitrogen Corporation); 4 h after the beginning of the incubation, the medium was replaced with standard culture medium described above.

The viability of the UM-UC-3P and UM-UC-3R cells were assessed using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [21]. Briefly, 4×10^4 cells were seeded in each well of 24-well plates and allowed to attach overnight. Cells were then treated with various concentrations of chemotherapeutic agents (methotrexate, paclitaxel, doxorubicin, cisplatin and gemcitabine) and/or OGX-011 or control ODN for 2 days. After 3 days of incubation, 250 μL (0.5 mg/mL) of MTT (Sigma Chemical Co.) in serum-free conditioned medium (Dulbecco's MEM/F12; Invitrogen Corporation) was added to each well. Formazan crystals were then dissolved in 200 μL of dimethyl sulphoxide. Absorbance was determined with a microculture plate reader (Bio-Tek Instruments) at 570 nm. Absorbance values were normalized to the values obtained from cells treated with oligofectamine alone to determine the percentage of survival. Each assay was performed in triplicate.

Apoptotic cells were identified by their subdiploid DNA content using flow cytometric analysis as described previously [21]. Briefly, cells were plated in 75-cm² dishes, and the day after, they were treated with ODN as described above. The cells were trypsinized 2 days after AS ODN treatment, washed with PBS, and were resuspended in 250 μL of 70% ethanol. After overnight incubation at 4 °C, cells were washed with PBS and then stained with 50 μg/mL of propidium iodide solution. After incubation for 30 min at room temperature, the stained cells were analysed for relative DNA content on a dual-laser flow cytometer (Beckman Coulter Epics Elite; Beckman, Inc., Miami, FL, USA). The proportion of cells undergoing apoptosis is defined as the

apoptotic index (AI). Each assay was performed in triplicate.

In the animal studies, ≈5 × 10^5 UM-UC-3P or UM-UC-3R cells were trypsinized, washed twice with PBS, and injected directly into the bladder wall of 6–8-week-old male athymic nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) via a 27-G needle under halothane anaesthesia. All animal procedures were performed according to the guidance of the Canadian Council on Animal Care and with appropriate institutional certification. Each experimental group consisted of nine mice. At 1 week after injection, mice were randomly selected for treatment with gemcitabine plus OGX-011 and gemcitabine plus control ODN. After randomization, 12.5 mg/kg of OGX-011 or control ODN was injected i.p. once daily for the first week, and three times per week thereafter. Gemcitabine was injected i.p (60 mg/kg) once weekly into each mouse for 3 weeks. At 5 weeks after the injection of tumour cells in the bladder wall, the mice were killed, and the presence of metastasis was examined in all abdominal organs. Death from multiple metastases during the treatment period was regarded as the endpoint of these experiments.

RESULTS

TREATMENT WITH GEMCITABINE UP-REGULATES sCLU-2 EXPRESSION LEVELS IN A TIME-DEPENDENT MANNER

Gemcitabine-sensitive UM-UC-3P cells were treated with 5 nM of gemcitabine, and whole cell lysates were harvested 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h after gemcitabine treatment. The whole cell lysate was harvested when cells were subconfluent. All protein samples were subjected to Western blot to detect changes in sCLU-2 protein expression levels. As shown in Fig. 1, treatment with 5 nM of gemcitabine induced up-regulation of sCLU-2 expression 12 h after gemcitabine treatment; however, after one passage in normal media, sCLU-2 expression levels decreased to levels similar to treatment naive UM-UC-3 cells.

BASELINE LEVELS OF sCLU-2 ARE UP-REGULATED IN GEMCITABINE-RESISTANT UM-UC-3R

The gemcitabine-resistant cell line, UM-UC-3R was developed by continued exposure to gemcitabine, whose concentration was serially increased up to 50 nM during >20 passages. After four passages of gemcitabine treatment up-regulated sCLU-2 expression increased 2.3-fold in UM-UC-3 cells compared with UM-UC-3P cells. sCLU-2 expression levels increased 6.5-fold in established UM-UC-3R cells, after short-term treatment with 50 nM gemcitabine, showing that sCLU-2 was still stress-induced in these resistant cells (Fig. 2A,B).

FIG. 1. Gemcitabine up-regulates sCLU-2 expression levels in a time-dependent manner. Gemcitabine-sensitive UM-UC-3P cells were treated with 5 nM of gemcitabine. Whole cell lysates were harvested after 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h. Treated cells were passed into a new 10-cm dish and cultured with complete media without gemcitabine. All protein samples were subjected to Western blot to detect changes in sCLU-2 protein expression levels.

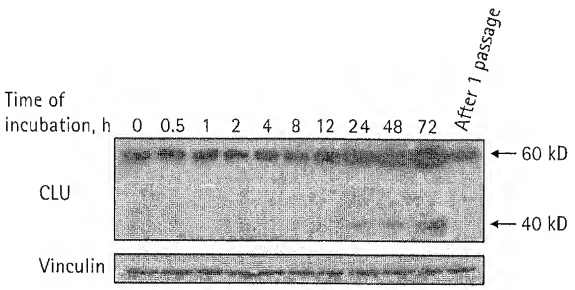
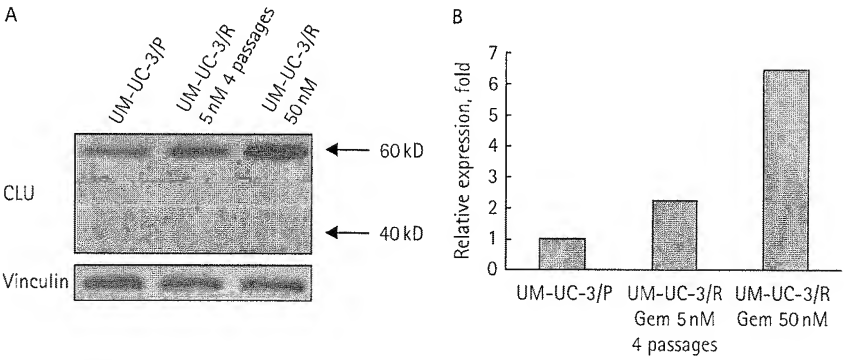


FIG. 2. sCLU-2 levels are increased in gemcitabine-resistant UM-UC-3 cells (UM-UC-3R). A,B, Gemcitabine resistant cell line UM-UC-3R was developed by serial and then continued exposure to gemcitabine (final concentration; 50 nM) for >20 passages. After 2 weeks culture in media without gemcitabine, baseline levels of sCLU-2 were assessed by Western blot, comparing UM-UC-3P to that of UM-UC-3 cells after four passages of initial treatment with gemcitabine. Established UM-UC-3R cells exhibited a further 6.5-fold increase in sCLU-2 expression levels.



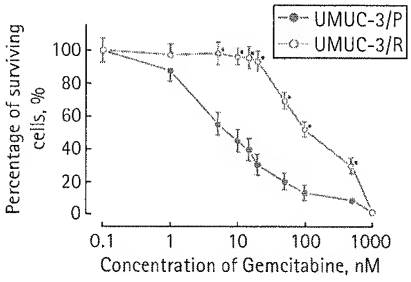
UM-UC-3R CELLS ARE RESISTANT TO CHEMOTHERAPEUTIC TREATMENT *IN VITRO*

To confirm the acquisition of resistance to gemcitabine in UM-UC-3R cells, an *in vitro* survival assay was performed. After 72 h of treatment with gemcitabine, the concentration that reduced the effect by 50% (IC_{50}) of UM-UC-3P cells was 10 nM, but was 10-fold higher in UM-UC-3R cells at 100 nM (Fig. 3). To analyse whether UM-UC-3R cells developed multidrug cross-resistance, UM-UC-3P and UM-UC-3R cells were treated with increasing concentrations of methotrexate, paclitaxel, doxorubicin or cisplatin using the same schedule described above. As shown in Table 1, the IC_{50} of UM-UC-3R cells increased by 1.5-, 4.2-, 2.5- and 3.0-fold, respectively, compared with those of UM-UC-3P cells.

OGX-011 INHIBITS sCLU-2 EXPRESSION IN BOTH UM-UC-3P AND UM-UC-3R CELL LINES IN A SEQUENCE-SPECIFIC AND DOSE-DEPENDENT MANNER

To determine whether OGX-011 inhibits sCLU-2 expression levels in UM-UC-3P and UM-UC-3R cells lines, both cell lines were treated with various concentrations of OGX-011 and control ODN. sCLU-2 expression in UM-UC-3P

FIG. 3. UM-UC-3R cells are resistant to chemotherapeutic treatment in vitro. *UM-UC-3P and UM-UC-3R cells were treated with various concentrations of gemcitabine for 72 h and cell viability was determined using MTT assay in each cell line. Each assay was performed in triplicate. *P < 0.01 vs UM-UC-3P (Student's* t*-test).* bars, SD.

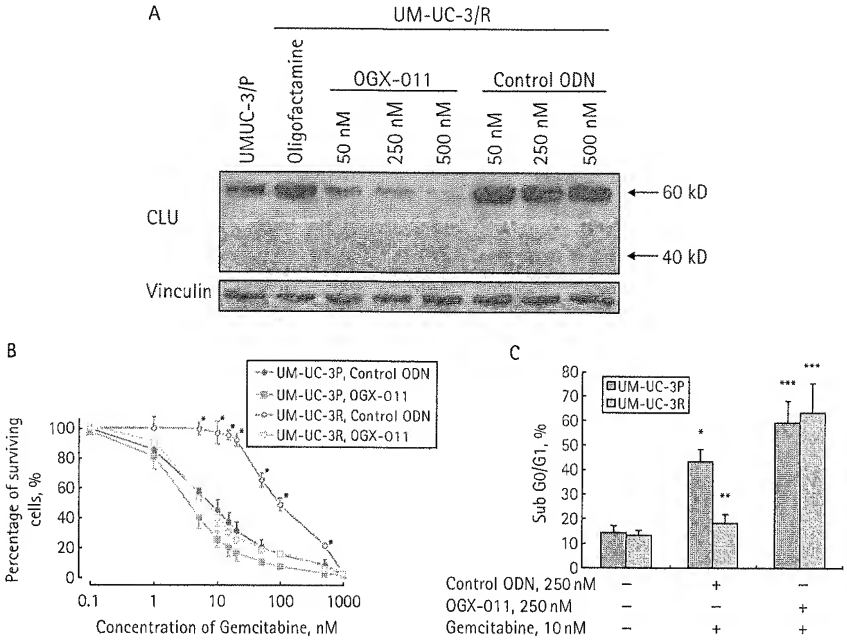


cells was suppressed by treatment with OGX-011 in dose-dependent and sequence-specific manners (data not shown). sCLU-2 expression in UM-UC-3R cells was also significantly suppressed by treatment with OGX-011 in dose-dependent and sequence-specific manners, suggesting that OGX-011 can inhibit sCLU-2 expression up-regulated through the development of the drug-resistant phenotype (Fig. 4A).

OGX-011 CHEMOSENSITIZES BOTH UM-UC-3P AND UM-UC-3R CELLS AND INDUCES APOPTOTIC CELL DEATH *IN VITRO*

To assess the effects of combined treatment with OGX-011 and gemcitabine, UM-UC-3P and UM-UC-3R cells were treated with OGX-011 or control ODN plus various concentrations of gemcitabine. OGX-011 marginally chemosensitizes UM-UC-3P cells to gemcitabine, reducing its IC_{50} from 10 nM to 5 nM. In contrast, the IC_{50} of gemcitabine in UM-UC-3R cells decreased more dramatically from 100 nM to 8 nM (Fig. 4B). Flow cytometric analysis showed that the AI after treatment with gemcitabine and control ODN significantly increased in UM-UC-3P cells compared with UM-UC-3R cells. The AI did not increase significantly in UM-UC-3R cells after treatment with gemcitabine and control ODN, indicating it was gemcitabine resistant. However, the AI significantly increased in both cell lines when treated with gemcitabine plus OGX-011. These results show that AS knockdown of sCLU-2 by OGX-011 reverses gemcitabine-resistance and sensitizes chemoresistant bladder cancer cells through the induction of apoptotic cell death.

FIG. 4. OGX-011-induced knockdown of sCLU-2 chemosensitized both UM-UC-3P and UM-UC-3R cells and increased apoptotic cell death in vitro. *A, Sequence-specific and dose-dependent inhibition of sCLU-2 expression by OGX-011 in UM-UC-3P and UM-UC-3R cell lines. UM-UC-3P and UM-UC-3R cells are treated with 250 nM of OGX-011 or control ODN for 2 days. Proteins were extracted and sCLU-2 expression levels were assessed by Western blot. Vinculin levels as loading controls. Oligofectamine, oligofectamine treated cells only. B, UM-UC-3P and UM-UC-3R cells are treated with 250 nM of OGX-011 or control ODN for 2 days, and then treated with various concentrations of gemcitabine for 2 days. Cell viability was determined by MTT assay. Each assay was performed in triplicate. *P < 0.01 vs other three treatment groups (Student's* t*-test).* bars, SD. *C, UM-UC-3P and UM-UC-3R cells are treated with 250 nM of OGX-011 or control ODN for 2 days, and then treated with or without 10 nM of gemcitabine for 2 days. Flow cytemetry was performed in triplicate to determine the rates of sub G0/G1 population.* bars, SD. **P < 0.01 vs no treatment group; **P < 0.01 vs UM-UC-3P cells treated with the same regimen; and ***P < 0.01 vs gemcitabine and control ODN treatment group (all Student's* t*-test).*



Agent	Concentration of IC_{50} UM-UC-3P	UM-UC-3R	IC_{50} increase (fold)*
Methotrexate	500 nM	750 nM	1.5
Paclitaxel	60 nM	250 nM	4.2
Doxorubicin	500 nM	1.25 μM	2.5
Cisplatin	25 μg/mL	75 μg/mL	3.0
Gemcitabine	10 nM	100 nM	10.0

TABLE 1 Cross-resistance of the UM-UC-3R cells to various cytotoxic agents

**The increase in IC_{50} was determined using MTT assay.*

SYSTEMIC ADMINISTRATION OF OGX-011 ENHANCES *IN VIVO* CYTOTOXIC EFFECT OF GEMCITABINE IN BOTH UM-UC-3P AND UM-UC-3R CELLS

We next tested the efficacy of combined gemcitabine and OGX-011 on tumour progression of UM-UC-3P and UM-UC-3R cell lines using orthotopic bladder cancer model *in vivo*. As shown in Table 2, the incidence of retroperitoneal or intra-abdominal lymph node metastasis, the incidence of haemorrhagic ascites, and the weight of primary tumour were significantly greater in mice bearing UM-UC-3R tumours treated with gemcitabine and control ODN compared with mice bearing UM-UC-3P tumours. Interestingly, although no mice

TABLE 2 Orthotopic tumour growth and changes in metastasis of UM-UC-3 sublines injected into the bladder wall of nude mice

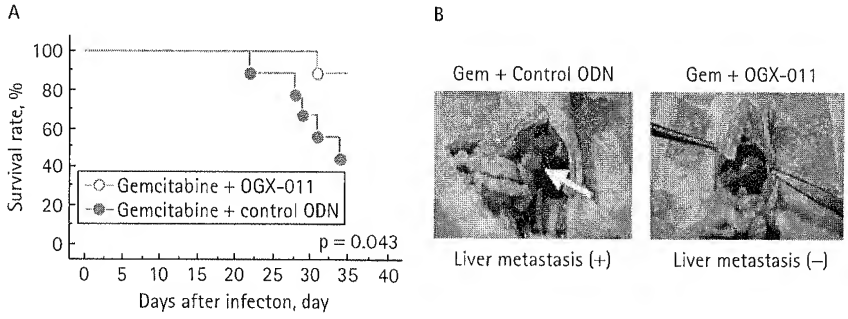
Variable	UM-UC-3P cells treated with*:		UM-UC-3R cells treated with*:	
	Gem + control ODN	Gem + OGX-011	Gem + control ODN	Gem + OGX-011
Incidence of metastasis, *n/N*				
Retropertioneal LN metastasis	6/9†	1/9	9/9§	3/9
Intra-abdominal LN metastasis	4/9†	0/9	9/9§	1/9
Liver metastasis	0/9†	0/9	6/9§	1/9
Incidence of haemorrhagic ascites	1/9†	0/9	7/9§	1/9
Mean (SD) weight of primary tumour, mg	175.2 (48.4)‡	70.2 (34.9)	367.9 (70.5)¶	101.9 (53.3)

*LN, lymph node; Gem, gemcitabine. *At 1 week after the implantation of tumour cells, 12.5 mg/kg of OGX-011 or control ODN was injected i.p. once daily for 1 week, and three times per week thereafter for 3 weeks; 60 mg/kg of gemcitabine was injected i.p. once a week for 3 weeks. †The incidence of metastasis or haemorrhagic ascites was significantly different from that in mice bearing UM-UC-3R tumors treated with the same regimen (P < 0.05, chi-square analysis). ‡The mean weight of the primary tumour was significantly different from that in mice bearing UM-UC-3R tumors treated with the same regimen P < 0.05, Student's t test). §The incidence of metastasis or haemorrhagic ascites was significantly different from that in mice bearing UM-UC-3R tumors treated with gemcitabine and OGX-011 regimen (P < 0.05, chi-square analysis). ¶The mean weight of the primary tumour was significantly different from that in mice bearing UM-UC-3R tumours treated with gemcitabine and OGX-011 regimen (P < 0.05, Student's t test).*

developed liver metastasis in the UM-UC-3P group, six mice bearing UM-UC-3R tumours developed macroscopic and multiple liver metastases. The addition of OGX-011, compared with control ODN, in combination with gemcitabine in the UM-UC-3R group significantly decreased the incidence of metastasis to lymph nodes, incidence of haemorrhagic ascites, and the weight of primary tumour.

All mice were killed on day 35. During this 35-day treatment period, there was no disease-related deaths in UM-UC-3P group. One mouse in UM-UC-3R group treated with gemcitabine and OGX-011, and five mice in UM-UC-3R group treated with gemcitabine and control ODN, died before day 35. All dead mice had extensive intra-abdominal dissemination of tumour and/or liver metastasis. As shown in Fig. 5A, survival in UM-UC-3R group was significantly prolonged after treatment with gemcitabine plus OGX-011 compared with control ODN ($P = 0.043$). Representative photographs of mice orthotopically injected with UM-UC-3R cells and treated with gemcitabine plus control ODN or OGX-011 are presented in Fig. 5B.

FIG. 5. Systemic administration of gemcitabine and OGX-011 prolonged survival of mice bearing UM-UC-3R tumours. A, About 5×10^6 UM-UC-3R cells were trypsinized, washed twice with PBS, and injected directly into the bladder wall of 6–8-week-old male athymic nude mice. At 1 week after injection, mice were randomly selected for treatment with gemcitabine plus OGX-011 and gemcitabine plus control ODN. After randomization, 12.5 mg/kg of OGX-011 or control ODN was injected i.p. once daily for 1 week, and three times per week thereafter. Gemcitabine was injected i.p. (60 mg/kg) once weekly into each mouse for 3 weeks. Each experimental group consisted of nine mice. The end point of this experiment was set at disease-related death before 5 weeks. At 5 weeks after the injection of tumour cells in the bladder wall, the mice were killed, and the presence of metastasis was examined in all abdominal organs. A, Kaplan–Meier survival curve of UM-UC-3R group. UM-UC-3R mice treated with gemcitabine and OGX-011 exhibited significantly prolonged survival rates compared with mice treated with gemcitabine and control ODN (P = 0.043, Mantel-Cox log-rank test). B, Representative photographs of mice orthotopically injected with UM-UC-3R cells and then treated with gemcitabine plus control ODN or OGX-011 were presented. Arrow, *macroscopic liver metastasis.*



DISCUSSION

Resistance to chemotherapeutic agents develops, in part, from alterations in the apoptotic machinery, due to increased activity of anti-apoptotic pathways, including stress-induced anti-apoptotic genes like *CLU* [16,22,23]. sCLU-2, also known as testosterone-repressed prostate message-2 or sulphated glycoprotein-2, has been shown to have important roles in various pathophysiological processes, such as reproduction, lipid transport, complement regulation and apoptosis [24]. As sCLU-2 expression increases in various benign and malignant tissues undergoing apoptosis, it has been regarded as a marker of cell death [25]. Recent studies have increasingly linked sCLU-2 up-regulation with stress-induced cytoprotection and treatment resistance in many cancer models [25–28], including bladder cancer [29]. sCLU-2 inhibition using OGX-011 has also been reported to sensitize treatment-naïve human bladder cancer models to cisplatin chemotherapy [29]. Moreover, we previously reported that sCLU-2 over-expression is closely associated with disease recurrence and progression in

patients with bladder cancer [14]. Accordingly, sCLU-2 has been regarded as an anticancer therapeutic target.

An initial objective of the present study was to characterize sCLU-2 expression levels in gemcitabine-naïve bladder cancer cells after exposure of sublethal doses of gemcitabine. The gemcitabine-sensitive human bladder cancer cell line, UM-UC-3, exhibited time-dependent increases of sCLU-2 expression after 12 h of gemcitabine treatment. This stress-induced sCLU-2 up-regulation is similar to that reported in previous studies using hormone-, radiation, or chemotherapy in various cancer models, implicating sCLU-2 as a stress-associated cytoprotective chaperone in cancer cells [25–30]. After short-term gemcitabine treatment sCLU-2 levels returned to basal levels. With continuous exposure to gemcitabine, UM-UC-3 cells developed a sustained 6.5-fold over-expression of sCLU-2 and a gemcitabine-resistant phenotype (UM-UC-3R) with a 10-fold increase IC_{50} of gemcitabine. Moreover, the IC_{50} of several other chemotherapeutic agents in UM-UC-3R cells also increased. Collectively, these findings suggest that treatment-induced increases in sCLU-2 expression is associated with the development of gemcitabine-resistance and the acquisition of multidrug resistant phenotype in bladder cancer.

The next objective of the present study was to investigate whether increased sCLU-2 levels in UM-UC-3R could be suppressed by OGX-011, the second generation AS ODN targeting the sCLU-2 gene, and whether sCLU-2 knockdown could enhance the activity of gemcitabine in UM-UC-3R cells, thereby reversing gemcitabine resistance. Treatment with OGX-011 significantly suppressed sCLU-2 levels in dose-dependent and sequence-specific manners in both UM-UC-3P and UM-UC-3R cells. Furthermore, OGX-011-mediated knockdown of sCLU-2 resulted in gemcitabine sensitization of both UM-UC-3P and UM-UC-3R cells, increasing apoptotic cell death even in gemcitabine-resisitant UM-UC-3R cells. We subsequently examined the *in vivo* effects of combined treatment with gemcitabine and OGX-011 on growth of UM-UC-3P and UM-UC-3R primary tumours, and metastases using an orthotopic approach. UM-UC-3R cells behaved more aggressively than UM-UC-3P cells with larger primary tumour weights and a higher incidence of metastases. The combined treatment with gemcitabine and OGX-011 significantly inhibited metastasis in UM-UC-3P cells, and this combined regimen also significantly delayed local and metastatic progression of UM-UC-3R cells, resulting in prolonged survival of mice bearing UM-UC-3R tumours. These findings indicate that targeting sCLU-2 using AS ODN synergistically delayed tumour progression in both gemcitabine-sensitive and -resistant cell lines.

In conclusion, the present study suggests that a gemcitabine-resistant phenotype in a human bladder cancer cell line was associated with increased sCLU-2 levels, and that OGX-011 suppressed this up-regulation and subsequently enhanced chemosensitivity in gemcitabine-resistant bladder cancer both *in vitro* and *in vivo*. This data provides preclinical proof of principle supporting the use of OGX-011 in second-line therapy combined with gemcitabine for advanced bladder cancer.

CONFLICT OF INTEREST

The University of British Columbia has submitted patent applications, listing Dr Gleave as inventor, on the antisense and siRNA sequences against clusterin. This IP has been licensed to OncoGenex Technologies, a Vancouver-based biotechnology company that Dr Gleave has founding shares in.

REFERENCES

1 **Thurman SA, DeWeese TL.** Multimodality therapy for the treatment of muscle-invasive bladder cancer. *Semin Urol Oncol* 2000; **18**: 313–22

2 **Studer UE, Bacchi M, Biedermann C *et al.*** Adjuvant cisplatin chemotherapy following cystectomy for bladder cancer: results of a prospective randomized trial. *J Urol* 1994; **152**: 81–4

3 **Freiha F, Reese J, Torti FM.** A randomized trial of radical cystectomy versus radical cystectomy plus cisplatin, vinblastine and methotrexate chemotherapy for muscle invasive bladder cancer. *J Urol* 1996; **155**: 495–500

4 **Skinner DG, Daniels JR, Russell CA *et al.*** The role of adjuvant chemotherapy following cystectomy for invasive bladder cancer: a prospective comparative trial. *J Urol* 1991; **145**: 459–67

5 **Stockle M, Meyenburg W, Wellek S *et al.*** Advanced bladder cancer (stages pT3b, pT4a, pN1 and pN2): improved survival after radical cystectomy and 3 adjuvant cycles of chemotherapy. Results of a controlled prospective study. *J Urol* 1992; **148**: 302–7

6 **Saxman SB, Propert KJ, Einhorn LH *et al.*** Long-term follow-up of a phase III intergroup study of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma: a cooperative group study. *J Clin Oncol* 1997; **15**: 2564–9

7 **Juffs HG, Moore MJ, Tannock IF.** The role of systemic chemotherapy in the management of muscle-invasive bladder cancer. *Lancet Oncol* 2002; **3**: 738–47

8 **Heinemann V.** Gemcitabine: progress in the treatment of pancreatic cancer. *Oncology* 2001; **60**: 8–18

9 **Stadler WM, Kuzel T, Roth B, Raghavan D, Dorr FA.** Phase II study of single-agent gemcitabine in previously untreated patients with metastatic urothelial cancer. *J Clin Oncol* 1997; **15**: 3394–8

10 **Moore MJ, Tannock IF, Ernst DS, Huan S, Murray N.** Gemcitabine: a promising new agent in the treatment of advanced urothelial cancer. *J Clin Oncol* 1997; **15**: 3441–5

11 **Grossman HB, Natale RB, Tangen CM *et al.*** Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med* 2003; **349**: 859–66

12 **von der Maase H, Hansen SW, Roberts JT *et al.*** Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of a large, randomized, multinational, multicenter, phase III study. *J Clin Oncol* 2000; **18**: 3068–77

13 **Vaughn DJ, Malkowicz SB.** Recent advances in bladder cancer chemotherapy. *Cancer Invest* 2001; **19**: 77–85

14 **Miyake H, Gleave M, Kamidono S, Hara I.** Overexpression of clusterin in transitional cell carcinoma of the bladder is related to disease progression and recurrence. *Urology* 2002; **59**: 150–4

15 **Miyake H, Eto H, Hara I, So A, Li D, Gleave ME.** Synergistic antitumor activity by combined treatment with gemcitabine and antisense oligodeoxynucleotide targeting clusterin gene in an intravesical administration model against human

bladder cancer kotcc-1 cells. *J Urol* 2004; **171**: 2477–81

16 **Lourda M, Trougakos IP, Gonos ES.** Development of resistance to chemotherapeutic drugs in human osteosarcoma cell lines largely depends on up-regulation of Clusterin/Apolipoprotein J. *Int J Cancer* 2007; **120**: 611–22

17 **Hoeller C, Pratscher B, Thallinger C *et al.*** Clusterin regulates drug-resistance in melanoma cells. *J Invest Dermatol* 2005; **124**: 1300–7

18 **Miyake H, Hara I, Kamidono S, Gleave ME, Eto H.** Resistance to cytotoxic chemotherapy-induced apoptosis in human prostate cancer cells is associated with intracellular clusterin expression. *Oncol Rep* 2003; **10**: 469–73

19 **Hara I, Miyake H, Gleave ME, Kamidono S.** Introduction of clusterin gene into human renal cell carcinoma cells enhances their resistance to cytotoxic chemotherapy through inhibition of apoptosis both *in vitro* and *in vivo*. *Jpn J Cancer Res* 2001; **92**: 1220–4

20 **Monia BP, Lesnik EA, Gonzalez C *et al.*** Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* 1993; **268**: 14514–22

21 **Yamanaka K, Rocchi P, Miyake H *et al.*** A novel antisense oligonucleotide inhibiting several antiapoptotic Bcl-2 family members induces apoptosis and enhances chemosensitivity in androgen-independent human prostate cancer PC3 cells. *Mol Cancer Ther* 2005; **4**: 1689–98

22 **Gleave M, Miyake H, Chi K.** Beyond simple castration: targeting the molecular basis of treatment resistance in advanced prostate cancer. *Cancer Chemother Pharmacol* 2005; **56** (Suppl. 1): 47–57

23 **Yang HH, Ma MH, Vescio RA, Berenson JR.** Overcoming drug resistance in multiple myeloma: the emergence of therapeutic approaches to induce apoptosis. *J Clin Oncol* 2003; **21**: 4239–47

24 **Rosenberg ME, Silkensen J.** Clusterin: physiologic and pathophysiologic considerations. *Int J Biochem Cell Biol* 1995; **27**: 633–45

25 **Sensibar JA, Sutkowski DM, Raffo A *et al.*** Prevention of cell death induced by tumor necrosis factor α in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). *Cancer Res* 1995; **55**: 2431–7

26 **Miyake H, Nelson C, Rennie PS, Gleave ME.** Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen independence in prostate cancer. *Cancer Res* 2000; **60**: 170–6

27 **Miyake H, Nelson C, Rennie PS, Gleave ME.** Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models. *Cancer Res* 2000; **60**: 2547–54

28 **Miyake H, Chi KN, Gleave ME.** Antisense TRPM-2 oligodeoxynucleotides chemosensitize human androgen-independent PC-3 prostate cancer cells both *in vitro* and *in vivo*. *Clin Cancer Res* 2000; **6**: 1655–63

29 **Miyake H, Hara I, Kamidono S, Gleave ME.** Synergistic chemsensitization and inhibition of tumor growth and metastasis by the antisense oligodeoxynucleotide targeting clusterin gene in a human bladder cancer model. *Clin Cancer Res* 2001; **7**: 4245–52

30 **Criswell T, Beman M, Araki S *et al.*** Delayed activation of insulin-like growth factor-1 receptor/Src/MAPK/Egr-1 signaling regulates clusterin expression, a pro-survival factor. *J Biol Chem* 2005; **280**: 14212–21

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Abbreviations: **MVAC**, methotrexate, vinblastine, doxorubicin, and cisplatin; **GC**, gemcitabine plus cisplatin; **sCLU-2**, clusterin; **AS**, antisense; **ODN**, oligodeoxynucleotide; **UM-UC-3P**, parental UM-UC-3 cells; **AI**, apoptotic index; **MEM**, Modified Eagle's Medium.